The PsaE Subunit Is Required for Complex Formation between Photosystem I and Flavodoxin from the Cyanobacterium *Synechocystis* sp. PCC 6803[†]

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ABSTRACT: The photoreduction of the oxidized and the semiquinone form of flavodoxin by photosystem I particles (PSI) from the wild type and a psaE deletion strain from the cyanobacterium *Synechocystis* sp. PCC 6803 was analyzed by flash-absorption spectroscopy to investigate a possible involvement of the PsaE subunit in this photoreduction process. The kinetics of the reduction of oxidized flavodoxin display a single-exponential component for both PSI preparations. Limiting electron transfer rates k_{obs} of ∼500 and ∼900 s^{-1} are deduced for the wild type and PSI from the psaE-less mutant, respectively, indicating that the PsaE subunit is not important for this photoreduction process. In the case of wild-type PSI, the reduction of flavodoxin semiquinone is a biphasic process, displaying a fast first-order phase with a $t_{1/2}$ of ∼13 μs which is then followed by a slower, concentration-dependent phase, for which a second-order rate constant k_2 of 2.2×10^8 M^{-1} cm^{-1} is calculated. In contrast, photoreduction of the semiquinone by PSI from the psaE-less mutant is monoexponential, displaying only one second-order component with a second-order rate constant similar to those observed for wild-type PSI ($k_2 = 1.5 \times 10^8$ M^{-1} cm^{-1}). The fast first-order component which is interpreted as an electron transfer process within a preformed complex between flavodoxin semiquinone and PSI is almost completely absent in the reduction of flavodoxin by the PsaE-less PSI. A similar loss of the fast phase is also observed for the photoreduction of flavodoxin semiquinone by PSI from a *Synechococcus elongatus* psaE-less mutant. Upon reconstitution of isolated PsaE to the PsaE-less PSI in vitro, ∼80% of the fast first-order kinetic component is recovered, indicating that PsaE is required for high-affinity binding of the flavodoxin semiquinone to PSI. In addition, chemical cross-linking assays show that flavodoxin can no longer be cross-linked to PSI in detectable amounts when PsaE is missing on the reaction center. Taken together, these experiments indicate that the PsaE subunit is required for complex formation between PSI and flavodoxin but is not required for an efficient forward electron transfer from photosystem I to both forms of flavodoxin.

During oxygenic photosynthesis, the photosystem I reaction center complex (PSI)[1] functions as a light-driven oxidoreductase, transferring electrons from the reduced periplasmic electron carrier proteins cytochrome c_6 or plastocyanin through the photosynthetic membrane to the soluble acceptor proteins ferredoxin or flavodoxin in the cytoplasm (see refs *1*−*3* for a review). Subsequently, ferredoxin and flavodoxin function as ubiquitous electron donors in a variety of metabolic processes such as $NADP^+$ reduction, carbon fixation, and nitrogen or sulfide assimilation (*4*, *5*). In general, [2Fe-2S] ferredoxins serve as the exclusive cytoplasmatic electron carrier proteins in land plant chloroplasts, and in cyanobacteria and most algae under normal conditions of growth. In addition, most cyanobacteria and algae contain flavodoxin as an alternative acceptor protein. Its physiological role is to substitute for ferredoxin under conditions of iron deprivation or stress (*5*, *7*−*9*). In most cyanobacteria and algae studied so far, flavodoxin is expressed in detectable amounts only under these conditions (*5*, *7*, *8*, *10*−*12*), although exceptions exist (*13*).

Flavodoxins are FMN-containing, low-molecular weight flavoproteins that are widely distributed throughout the bacterial kingdom, serving as mediators for low-potential electron transfer reactions (*14*). In contrast to [2Fe-2S] ferredoxins, however, flavodoxins are capable of performing two subsequent one-electron transfer reactions, thus shuttling the redox-active FMN group between an oxidized form (Fld), a thermodynamically stabilized protonated semiquinone ($FldH^•$), and a fully reduced hydroquinone form ($FldH^-$) (*15*). The corresponding redox potentials are in the −180/−240 mV region for the Fld/$FldH^•$ couple (at pH 7) and in the −370/−470 mV region for the $FldH^•$/$FldH^-$ couple for the long-chain flavodoxins found in phototrophic organisms (*16*). Since the latter value is very similar to that of the redox couple of ferredoxin, it is generally assumed that flavodoxins shuttle between the semiquinone and the fully reduced forms under the physiological conditions of the cell (*7*). Substantiating their role as ferredoxin substitutes, cyanobacterial flavodoxins have been shown to react efficiently with PSI,

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[1] Abbreviations: PSI, photosystem I; PsaE, subunit IV of photosystem I; psaE, gene encoding subunit IV of photosystem I.

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ferredoxin:$NADP^+$ oxidoreductase (FNR), nitrogenase, and hydrogenase (*17*−*20*; see refs *5* and *7* for a review).

Detailed spectroscopic analyses of the photoreduction of flavodoxin by photosystem I have shown that the oxidized and the semireduced forms of flavodoxin are reduced by PSI by different reaction mechanisms (*19*, *21*). While a simple bimolecular mechanism is observed for the reduction of oxidized flavodoxin, the photoreduction of the semiquinone is biphasic and includes a first-order phase that is indicative of an intramolecular electron transfer reaction within a tight complex between PSI and the flavodoxin semiquinone. Cross-linking studies indicate that the low-molecular weight PSI subunits PsaC, PsaD, PsaE, and PsaF are involved in binding flavodoxin to photosystem I (*22*). In addition, the flavodoxin docking site has been visualized by electron microscopy, showing that flavodoxin binds to the cytoplasmic site of PSI at a position close to the extrinsic PSI subunits PsaC, PsaD, and PsaE (*23*), a region very similar to the one occupied by ferredoxin (*24*). With respect to ferredoxin, detailed studies have shown that ferredoxin is in close contact with the PsaD subunit (*25*−*27*). In addition, spectroscopic analyses have shown that the PsaE subunit is involved in the binding of ferredoxin to PSI (*28*). In a recent study, the PsaE subunit, which is dispensable for photoautotrophic growth in cyanobacteria (*29*, *30*), was shown to be not essential for the photoreduction of flavodoxin and $NADP^+$ (*31*). In this study, we report a detailed spectroscopic analysis of flavodoxin photoreduction by PSI from the wild type and a psaE deletion strain of the cyanobacterium *Synechocystis* sp. PCC 6803 and show that the PsaE subunit appears to be strictly required for an efficient binding of flavodoxin to photosystem I.

MATERIALS AND METHODS

Biological Material. Photosystem I complexes from the *Synechocystis* sp. PCC 6803 wild type and the psaE deletion mutant FKE_2 (*28*) were purified as described previously (*32*). PSI preparations from the *Synechococcus elongatus* wild type and the psaE deletion strain ES/8 (U. Mühlenhoff, unpublished) were obtained following the protocol described in ref *33*. Flavodoxin was purified either from *Synechocystis* sp. PCC 6803 as described previously (*11*) or by overexpression of the flavodoxin-encoding isiB gene in *Escherichia coli*. For recombinant expression, a genomic fragment from *Synechocystis* sp. PCC 6803 carrying the isiA/B operon (*34*) was modified by PCR in the presence of primers isiB-N (5′-ACATATGACAAAAATTG-3′) and isiB-C (5′-TCGAATCCAATGGATCC-3′) to create a *Nde*I restriction site at the start codon of the isiB gene, and the modified gene was subsequently inserted into the *Nde*I and *Bam*HI sites of the expression vector pET3a (*35*). Flavodoxin was expressed in *E. coli* strain BL21(DE3) upon induction with 0.7 mM IPTG; cells were harvested 6 h after induction, and flavodoxin was subsequently purified and fully reconstituted with the FMN cofactor essentially as previously described (*22*, *23*, *36*). Flavodoxin from *Synechococcus* sp. PCC 7002 was isolated as described in ref *22*.

PsaE from *Synechocystis* sp. PCC 6803 and *S. elongatus* were overexpressed in *E. coli.* For expression, the psaE gene from *Synechocystis* sp. PCC 6803 (*29*) was amplified from genomic DNA by PCR in the presence of the primers fpg-N (5′-TTTCCCTAGAACCATCTCCCAGGAG-3′) and fpg-C (5′-GCCTGGTAGGAGCCAGACTTTGCC-3′), and its start codon was modified by PCR in the presence of the fpg-C and a 6803psaE-N primer (5′-ACCATGGCCTTAAATCG-3′) to harbor a *Nco*I restriction site. In addition, a TaqI fragment carrying psaE from *S. elongatus* (*37*) was modified by PCR in the presence of the M13 reverse and a psaE-N primer (5′-ATAACCCATGGTGCAACGTG-3′) to harbor a *Nco*I site at the start codon of psaE. The genes from both strains were each inserted into the *Nco*I and *Xho*I sites of the expression vector pET15b (*35*). Both proteins were expressed in the form of inclusion bodies in *E. coli* strain BL21(DE3) upon induction with 1 mM IPTG, and the proteins were solubilized and purified by gel filtration essentially as described previously (*30*).

Chemical Cross-Linking, Reconstitution Assays, Immunoblot Analysis, and Photoreduction Assays. Isolated photosystem I particles were cross-linked to flavodoxin using the zero-length cross-linking agent *N*-ethyl-3-(diaminopropyl)-carbodiimide (EDC) as described previously (*22*, *25*): PSI at a final concentration of 75 μg of chlorophyll/mL in 20 mM Mops (pH 6.5), 5 mM $MgCl_2$, and 0.03% β-dodecyl maltoside (β-DM) was treated in the presence of 10 μM flavodoxin with 3 mM EDC for 30 min at 25 °C. The reactions were terminated by addition of ammonium acetate (pH 7.8) to a final concentration of 100 mM on ice, and the cross-linked complexes were subsequently recovered by centrifugation in linear 0.2 to 0.8 M sucrose gradients in 50 mM Tris-HCl (pH 8.3) supplemented with 0.03% β-DM at 150000*g* for 16 h in a SW 40.1 rotor (Beckman Instruments). The PsaE subunit was reconstituted using the protocol previously described (*28*): PSI particles of the psaE-less mutants were incubated at a final concentration of 100 μg of chlorophyll/mL in the presence of 100 μM recombinant PsaE in 50 mM TRIS (pH 8.5), 1 mM EDTA, and 0.03% β-DM for 60 min at 25 °C, and the PSI complexes were subsequently recovered by centrifugation in sucrose gradients as described above. SDS−polyacrylamide gel electrophoresis was carried out using 12.5% polyacrylamide gels (*38*). Samples equivalent to 4 μg of chlorophyll were applied per lane. The separated polypeptides were electrotransferred to nitrocellulose filters and immunodecorated with antibodies against *Synechococcus* sp. PCC 7002 flavodoxin using standard protocols (*39*). The blots were subsequently incubated with anti-rabbit-IgG antibodies linked to horseradish peroxidase, and visualization was carried out by enhanced chemiluminescence detection (ECL, Amersham). Antibodies were raised at the Institut for Immunobiology at the University of Freiburg. Flavodoxin photoreduction was performed with isolated PSI complexes in the presence 5 μM flavodoxin from *Synechocystis* sp. PCC 6803 as described previously (*22*).

Flash-Absorption Spectroscopy. Flash-induced absorption changes at 580 and 820 nm were measured at 296 K on a single-beam spectrophotometer essentially as described previously (*40*). Flash excitation was performed using a dye laser at 695 nm (pyridine in ethanol, 8 nm fwhm, 20% conversion efficiency, UDL110 Lasertechnik Berlin) pumped with a frequency-doubled Nd:YAG laser (5 ns fwhm). The measuring wavelength was selected with a monochromator (Jobin Y von H25) in front of the square cuvette (sample volume of 1 cm × 1 cm × 1 cm) and an interference filter

(4.2 or 9 nm fwhm, for measurement at 580 or 820 nm, respectively) in front of the detecting photodiode (1 cm^2). The position of the optical grating of the monochromator was adjusted to the maximal transmission of the interference filter, and the slits were opened to 4 mm, corresponding to 6 nm full width at half-maximum. For some experiments at 580 and 820 nm, additional blue-green glass filters BG 40/5 and RG 780/3 (Schott) were used for complete suppression of the laser artifact. Signal disturbance by the laser artifact was monitored without measuring light. The duration of the laser artifact was typically shorter than 4 μs. To minimize actinic effects of the measuring light during measurements at 580 nm, a shutter was placed in front of the cuvette and opened 4 ms before flash excitation. Multiple flash experiments were routinely made with a repetition rate of 0.1 Hz, a rate experimentally determined to be sufficiently low to allow a complete return to equilibrium. In all experiments, flashes of saturating intensity were used.

For flash-absorption experiments, PSI reaction centers were suspended at a standard concentration of 0.35 μM (varying $\pm$0.05 μM for different samples) in 50 mM Mops (pH 7.0) supplemented with 0.03% β-DM, 5 mM $MgCl_2$, 20 μM DPIP (dichlorophenolindophenol), and 2 mM sodium ascorbate. In addition, 340 μM glucose 6-phosphate, 0.25 unit/mL glucose-6-phosphate dehydrogenase, and 0.01 unit/mL spinach ferredoxin:$NADP^+$ oxidoreductase (FNR) were added. These components are required for the in situ generation of flavodoxin semiquinone (see below). For standard assays, flavodoxin was present at a final concentration of 10 μM and the sample volume was 1 mL. For measuring the reduction of oxidized flavodoxin, flavodoxin was added to the sample in complete darkness and absorption changes up to 14 ms were recorded using a single flash and an electrical bandwidth of 100 kHz. Afterward, $NADP^+$ (20 μM) was added to the cuvette to start the enzymatic reduction of flavodoxin to its semiquinone state. Complete transformation to the semiquinone required about 10 min and resulted in a stable absorption of the sample at 580 nm as judged by the transmitted light intensity measured with the same setup. Following the complete reduction, the absorption changes at 580 nm due to the photoreduction of the semiquinone (second wave) were then measured by repetitive flash-exitation experiments with an electrical bandwidth of 1 MHz up to 8 ms. Ten to 60 individual signals were averaged for the kinetic traces used in the analysis. To compare the signals of samples with slightly different PSI concentrations, flash-induced absorption changes at 820 nm were recorded repetitively (five scans) up to 160 ms with an electrical bandwidth of 30 kHz. Control experiments were carried out in the absence of flavodoxin and $NADP^+$ at 580 and 820 nm under the same experimental conditions.

Analysis of Kinetic Measurements. Absorption transients were fitted to a sum of one- or two-exponential components and a constant offset with the program GNUPLOT (Unix version 3.5, Williams), performing nonlinear least-squares fitting using the Marquart−Levenberg algorithm. The fit was performed in a time range where transient signals are due to the photoreduction of flavodoxin, i.e., from 3−4 μs up to 8 ms for the reduction of the semiquinone or 0.03 ms up to 14 ms after the laser flash for the reduction of oxidized flavodoxin.

The amount of flavodoxin photoreduced in a sample was calculated from the absorption changes observed at times when all transient signals of the reaction have reached their maximum value as they result from a fit. In the case of flavodoxin semiquinone, this amplitude is given by the fitted constant offset which represents the entire absorption change after the exciting laser flash, i.e., the absorption changes due to charge separation between $P700^+$ and $(F_A/F_B)^-$ and those due to electron transfer to flavodoxin semiquinone. From this amplitude, the contribution due to charge separation between $P700^+$ and $(F_A/F_B)^-$ as determined from control experiments performed in the absence of flavodoxin was subtracted after correction for slightly different PSI concentrations. For this purpose, kinetic traces recorded in the presence or absence of flavodoxin were scaled to the same PSI concentration using scaling factors determined from comparison of the absorption changes (between 50 and 200 μs after the laser flash) measured at 820 nm for each sample. In the case of oxidized flavodoxin, the amount of semiquinone formed was determined directly from the fitted amplitude assuming a monoexponential decay. Flavodoxin concentrations were then calculated assuming extinction coefficients ϵ_{580} of 3900 M^{-1} cm^{-1} for flavodoxin (*7*) and 630 M^{-1} cm^{-1} for $(F_A/F_B)^-$ (*41*). The amount of flavodoxin that was actually reduced in a sample was then compared to the total amount of PSI present in the cuvette as calculated from the initial absorption change determined at 820 nm assuming an extinction coefficient ϵ_{820} of 6500 M^{-1} cm^{-1} for *Synechocystis* sp. PCC 6803 and *S. elongatus* (*42*). To compare absorption changes in different samples, signals were adjusted to the same PSI concentration as described for the scaling of the control experiments.

RESULTS

The photoreduction process of both the oxidized and the semireduced form of flavodoxin was studied by monitoring the flash-induced absorption changes observed at 580 nm in the presence of PSI preparations from the *Synechocystis* sp. PCC 6803 wild type and the psaE-less mutant FKE_2 as shown in Figure 1. As described previously, the measuring wavelength of 580 nm is suitable for observing absorption changes due to reduction and/or oxidation of flavodoxin since it corresponds to the absorption maximum of the semiquinone form of the FMN cofactor (*7*, *21*). Panels A−D of Figure 1 each show the absorption changes elicited by a saturating laser flash in PSI preparations from either the wild type or the psaE-less mutant in the presence of flavodoxin (solid lines) or its absence (dotted lines) under two different experimental setups that are required for the separate observation of the photoreduction of the oxidized and semireduced forms of flavodoxin (*21*). To allow the direct comparison of the absorption changes for all samples, flavodoxin was present at a standard concentration of 10 μM and all traces shown were scaled to a PSI concentration of 0.33 μM as determined from the absorption change at 820 nm (see Materials and Methods). In the absence of flavodoxin, light excitation of PSI results in charge separation between $P700^+$ and the terminal acceptor, either F_A or F_B (*43*). This process is monitored by a fast step decrease of the absorption at 580 nm, taking place within a few hundred nanoseconds (Figure 1, dotted lines of panels A and C for the wild type and panels B and D for PsaE-less PSI).

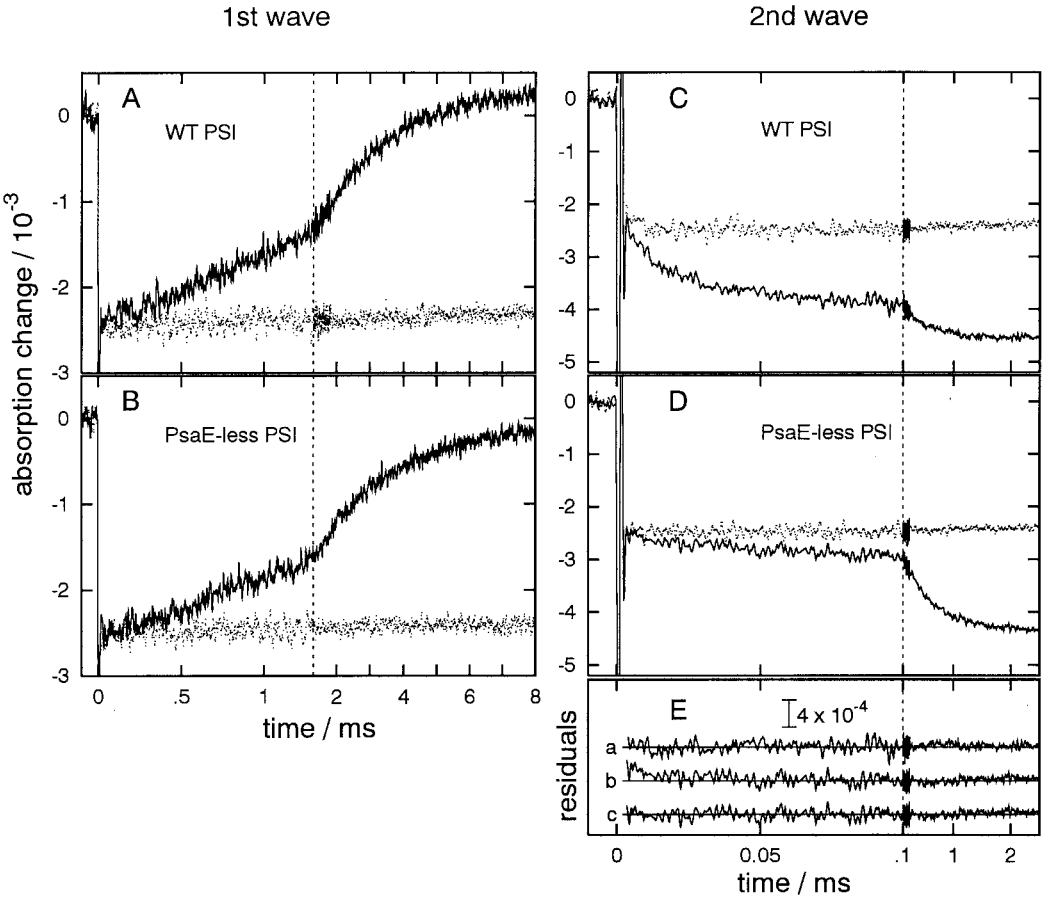

FIGURE 1: Flash-induced absorption changes measured at 580 nm with isolated photosystem I complexes from the *Synechocystis* sp. PCC 6803 wild type (panels A and C) or the psaE-less mutant FKE_2 (panels B and D) in the presence and absence of flavodoxin. (A and B, solid lines) Traces of single-flash experiments recorded in the presence of 10 μM oxidized flavodoxin from *Synechocystis* sp. PCC 6803. (C and D) Averaged traces of multiple-flash experiments recorded in the presence of 10 μM flavodoxin semiquinone from *Synechocystis* sp. PCC 6803 (solid lines, 20 scans). Dotted lines in panels A−D correspond to traces of control experiments recorded on the same PSI preparations but in the absence of flavodoxin (10 scans). All samples contained isolated PSI at 0.34 ± 0.03 μM (as determined from the absorption changes at 820 nm), and the traces shown were adjusted for slightly differing PSI concentrations (see Materials and Methods). In all traces, the vertical dashed line indicates a switch to a higher sampling rate. (E) Residuals for fits of the traces for semiquinone reduction by PSI complexes from the wild type and the psaE-less mutant shown in panels C and D. Trace a is the trace shown in panel C for wild-type PSI, fitted according to a biphasic decay. Trace b is the trace of panel D for PsaE-less PSI, fitted according to a monophasic decay. Trace c is the trace of panel D, fitted according to a biphasic decay (for details, see the text).

Afterward, practically no further absorption changes are observed during the first 10 ms following the laser flash for PSI from *Synechocystis* sp. PCC 6803 except for a slow decay which can be attributed to a recombination event between $P700^+$ and $(F_A/F_B)^-$ taking place in PSI in the absence of external acceptors (*41*, *43*, *44*).

Reduction of Oxidized Flavodoxin. For observing the photoreduction of the oxidized form of flavodoxin by PSI, the first wave of the photoreduction, flavodoxin was added (in excess compared to PSI) to the sample cuvette in complete darkness. In the presence of oxidized flavodoxin, the flash-induced initial absorption decrease is followed by an absorption increase which can be ascribed to the reduction of oxidized flavodoxin by $(F_A/F_B)^-$ (solid lines in panels A and B of Figure 1 for the wild type and the PsaE-less PSI, respectively). Like the results obtained with PSI from *Synechococcus* sp. PCC 7002, this process is apparently monophasic for PSI from both the wild type and the psaE-less mutant of *Synechocystis* sp. PCC 6803, displaying only one concentration-dependent phase with a $t_{1/2}$ of ~2 ms at a flavodoxin concentration of 10 μM. Direct comparison of the traces in panels A and B of Figure 1 reveals no obvious different behavior for PSI from the wild type (panel A, solid line) and the psaE-less mutant (panel B, solid line), and a comparable amount of 0.80 and 0.70 molecule of oxidized flavodoxin are reduced per PSI reaction center from the *Synechocystis* sp. PCC 6803 wild type and the psaE-less mutant, respectively (see Materials and Methods for details). In addition, PSI particles from the wild type and the psaE-less mutant exhibit comparable steady state flavodoxin photoreduction rates of 87.9 and 95.6 μmol of flavodoxin (mg of chlorophyll)$^{-1}$ h^{-1}, respectively, and chlorophyll/P700 ratios of ~75 were determined for PSI preparations from both strains. Taken together, these results uniformly indicate that PsaE is not required for the photoreduction of oxidized flavodoxin.

Reduction of Flavodoxin Semiquinone. For measurement of the reduction of the flavodoxin semiquinone, i.e., the second wave of the photoreduction process, flavodoxin must be reduced to its semiquinone form prior to the spectroscopic investigation. The previously employed technique for generating the semiquinone which was based on preillumination of the sample in the cuvette (*21*), however, needed to be improved as it did not allow for full control of the amount

of semiquinone present in the cuvette. In addition, the time available for flash-absorption experiments was restricted by the fact that flavodoxin semiquinone is slowly reoxidized by air (*15*, *21*). Therefore, an enzymatic reaction for the reduction of flavodoxin in situ was devised which is based on the observation that flavodoxin can accept electrons from FNR in a reversed electron transfer reaction similar to the reversed electron flow to ferredoxin found in heterocyst cells. For achieving this reduction to the semiquinone in situ, a pool of reduced NADPH is formed by glucose-6-phosphate dehydrogenase in the presence of glucose 6-phosphate and $NADP^+$ and the electrons flow from reduced NADPH via FNR to flavodoxin (see Materials and Methods). At pH 7.0, flavodoxin is quantitatively converted to the semiquinone form within 10 min after addition of $NADP^+$ (at 10 μM flavodoxin) and remains stable for at least 30 min, thus allowing the photoreduction of the semiquinone to be studied by repetitive flash experiments (typically 20 scans at a repetition rate of 0.1 Hz). A detailed description of this process will be given elsewhere.

Flash-induced absorption changes of flavodoxin semiquinone in the presence of either wild-type PSI (Figure 1C, solid lines) or the PsaE-less PSI (Figure 1D, solid lines) are reported on a shorter time scale than for the first wave (see the figure legend). At this time resolution, a bleaching is observed up to approximately 4 μs in the samples which is due to a laser artifact not completely suppressed by the interference filter in front of the detector. As expected, the initial bleaching is followed by a further absorption decrease at 580 nm which is characteristic of the photoreduction of flavodoxin semiquinone to $FldH^-$ (*21*). In the presence of wild-type PSI, a biphasic decay is observed consisting of a fast, first-order kinetic component ($t_{1/2} \sim 13$ μs) which is followed by a slower, concentration-dependent phase (Figure 1C). In the presence of PSI from the psaE-less mutant (Figure 1D), however, the fast kinetic component, which is attributed to electron transfer from PSI to flavodoxin within a preformed complex and which makes up about 60% of the entire amplitude in the case of wild-type PSI at 10 μM flavodoxin, is almost completely absent. The reduction of the semiquinone is thus almost monophasic, displaying only one concentration-dependent phase with a rate of 1.7 ms^{-1} (at a flavodoxin concentration of 10 μM), a rate comparable to the one observed for the wild type (see Table 1). Nevertheless, when traces like the ones shown in Figure 1D are fitted assuming a simple monoexponential decay, a small but significant deviation is observed for the PsaE-less PSI which may be partly due to a residual amount of a fast phase (Figure 1E, trace b). To estimate the upper limit for such a residual phase, the transients were fitted to a biphasic decay similar to those of the wild type (time range from 3.5 or 4 μs for the mutant and the wild type, respectively, to 8 ms). However, in the case of the PsaE-less PSI, the amplitude of the fast components is too small to allow the determination of its parameters by an unrestricted fit; thus, the half-time of the fast phase was fixed at 13 μs, a value that results from the fit for the wild type. The resulting residuals of these fits shown in panel E of Figure 1 (traces a and c) indicate satisfactory results for both the wild type and the mutant. According to these fits, the 60% of the total fitted amplitude that is due to a fast phase in the case of the wild type corresponds to less than 10% in the PsaE-less PSI.

Table 1: Kinetic Characteristics of Flavodoxin Semiquinone Reduction Observed for Different Photosystem I Complexes under Standard Conditions[a]

source of PSI particles	contribution of the fast phase to the total amplitude (%)	observed rate of the slow phase (ms^{-1})	flavodoxin molecules reduced per PSI particle
Synechocystis sp. PCC 6803 wild type	60	2.6	0.85
Synechocystis sp. PCC 6803 psaE$^-$ mutant	<10	1.7	0.78
6803 psaE$^-$ mutant reconstituted with 6803 PsaE	50	2.5	0.79
6803 psaE$^-$ mutant reconstituted with *S. elongatus* PsaE	80	7.7	0.67
S. elongatus wild type	30	2.8	0.88
S. elongatus psaE$^-$ mutant	0	0.3	0.65

[a] Standard experiments were performed in the presence of PSI at 0.35 and 10 μM flavodoxin semiquinone from *Synechocystis* sp. PCC 6803. The traces were fitted to a biphasic decay (see Materials and Methods).

Taking into account the fact that the value obtained for the PsaE-less PSI is an upper limit, as there may be a small amount of additional fast-decaying signals due, for instance, to antenna triplet states, one can state that the reduction of the semiquinone is in fact monophasic. Finally, when the amount of flavodoxin semiquinone that is reduced by PSI from the *Synechocystis* sp. PCC 6803 wild type and the psaE-less mutant is calculated, comparable values of 0.85 and 0.78 flavodoxin molecule are found for PSI from the wild type and the mutant strain, respectively (Table 1, see Materials and Methods for details). Thus, the efficiency of electron transfer to flavodoxin semiquinone is almost identical for both photosystems.

Quantitation of the Processes. For a more detailed quantification of the two photoreduction processes, the concentration dependencies of the kinetic characteristics observed for PSI from the wild type and the psaE-less mutant were compared. Traces as shown in Figure 1 were recorded for flavodoxin concentrations ranging from 1 to 20 μM in excess over PSI concentrations. For analyzing the first wave, data from single-flash experiments were fitted with one exponential component (range of 0.03−14 ms), and for the second wave, traces were fitted with two exponentials in a range from 4 μs to 8 ms. For the first wave, the plot of the observed rate constants versus the flavodoxin concentration shown in the upper part of Figure 2 reveals only a slight difference between the mutant (open circles) and the wild-type photosystem (closed circles). In both cases, the rate seems to approach an asymptotic value for k_{obs} of ~0.5 and 0.9 ms^{-1} for the wild type and the mutant, which correspond to half-times of 1.3 and 0.8 ms, respectively. For the second wave, the observed rate of the slower phase shows a linear increase with the concentration as expected for a second-order kinetic process (Figure 2, middle part). From the slope of a linear regression of the data, second-order rate constants k_2 of 2.2×10^8 and 1.5×10^8 M^{-1} s^{-1} can be derived for the wild type (closed circles, solid line) and the PsaE-less PSI (open circles, dashed line), respectively.

For the reduction of the flavodoxin semiquinone by PSI from the *Synechocystis* sp. PCC 6803 wild type, the fast

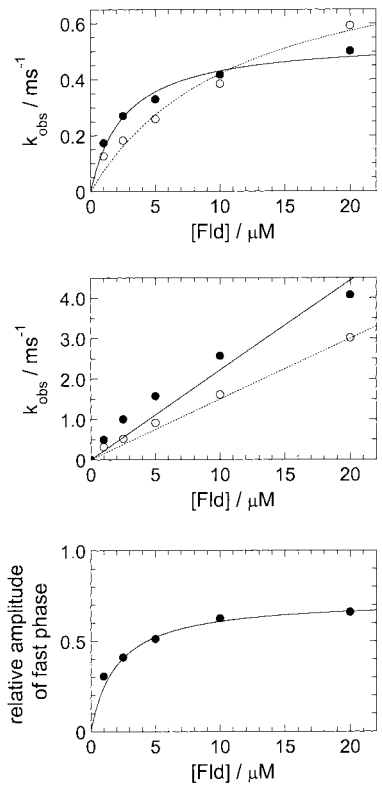

FIGURE 2: Concentration dependencies of the kinetic characteristics of the two waves of flavodoxin reduction by photosystem I complexes from the wild type and a psaE deletion strain of *Synechocystis* sp. PCC 6803. Traces similar to the ones shown in Figure 1 were recorded with flavodoxin concentrations ranging from 1 to 20 μM for wild-type PSI complexes (closed circles and solid lines) and PSI from the psaE-less mutant of *Synechocystis* sp. PCC 6803 (open circles and dashed lines): (top) the observed rate for the photoreduction of the oxidized flavodoxin, (middle) the observed rate of the slow phase for the photoreduction of the flavodoxin semiquinone, and (bottom) the relative amplitude of the fast phase ($t_{1/2} \sim 13$ μs) with respect to the sum of the two phases for semiquinone reduction by PSI from the wild type, as it results from fits of signals as in Figure 1 (panel C) with two exponentials. The continuous line is the result of a fit of the data in a simple model (see the text).

phase occurs with a half-time $t_{1/2}$ of $\sim$13 μs, a value that remains approximately constant for all flavodoxin concentrations investigated, indicating that this phase represents a first-order kinetic component. As shown in the lower part of Figure 2 (closed circles, straight line), its amplitude increases with increasing flavodoxin concentrations. According to a reaction mechanism model which assumes a simple binding equilibrium between PSI and flavodoxin, this amplitude accounts for the amount of stable complexes formed between the flavodoxin semiquinone and PSI (for details, see ref *21*). The data were fitted according to this model using the dissociation constant and the maximum relative amplitude as the free parameter (Figure 2, lower part). From the continuous curve shown in Figure 2 (lower part), a dissociation constant K_d of 2 μM for the wild type and a maximum amplitude of 0.73 are derived. To estimate the maximum contribution of a similar fast component for the PsaE-less PSI, the maximum amplitude of a possible fast phase was estimated by the procedure described above, and a slight increase of its amplitude from 0% for the lowest concentration (1 μM) up to 13% for the highest concentration was found (not shown). However, as the obtained data are rather

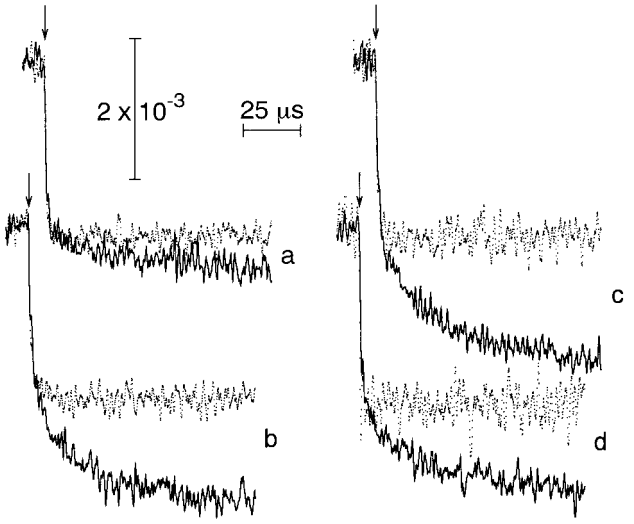

FIGURE 3: Flash-induced absorption changes at 580 nm in the presence of flavodoxin semiquinone from *Synechocystis* sp. PCC 6803 for different photosystem I particles from *Synechocystis* sp. PCC 6803: (a) the PsaE-less PSI complex from the psaE-less mutant, (b) wild-type PSI, (c) PSI from the psaE-less mutant reconstituted with PsaE from *S. elongatus*, and (d) PSI from the psaE-less mutant reconstituted with PsaE from *Synechocystis* sp. PCC 6803. Traces of parts a−d are the average of 40 experiments recorded in the presence of 10 μM flavodoxin semiquinone (solid lines) or 20 experiments (10 for traces in parts c and d) in its absence (dotted lines). Experiments were performed in a manner similar to that for the experiments depicted in Figure 1 (except for an additional filter for suppressing the laser artifact completely) and scaled to identical PSI concentrations. The signals shown are expanded to the time range of 100 μs, where the observable absorption change is mainly due to the fast first-order phase ($t_{1/2} \sim 13$ μs). The arrows indicate the time of the flash.

uncertain, a K_d value cannot be confidently derived from these data. The affinity for flavodoxin semiquinone is too drastically reduced in the PsaE-less PSI to allow the determination of quantitative data by standard assays.

In Vitro Reconstitution. To confirm the role of the PsaE subunit in the binding of flavodoxin semiquinone, PsaE from *Synechocystis* sp. PCC 6803 or *S. elongatus* was reconstituted to the PsaE-less PSI complexes in vitro using the protocol described previously (*28*), and the kinetic characteristics of semiquinone reduction by these reconstituted complexes were monitored by flash-absorption spectroscopy. Figure 3 shows traces elicited at 580 nm with reconstituted PSI complexes in the presence and absence of flavodoxin semiquinone on a 100 μs time scale. For all samples, a standard flavodoxin concentration of 10 μM was used and the signals were scaled to the same concentration of 0.33 μM P700 (same as for Figure 1) so they would be directly comparable. When the traces for semiquinone reduction by the PsaE-less PSI are compared before and after reconstitution with PsaE from *Synechocystis* sp. PCC 6803 (Figure 3, parts a and d), it is immediately apparent that a fast phase is recovered. Its relative amplitude corresponds to 50% of the entire amplitude, indicating that 83% of the fast phase was recovered upon in vitro reconstitution of the PsaE subunit (see Table 1). Qualitatively similar, the fast phase of semiquinone reduction is also recovered upon reconstitution of the PsaE subunit from *S. elongatus* to the PsaE-less PSI from *Synechocystis* sp. PCC 6803 (Figure 3, trace c). For this chimeric photosystem, however, a relative amplitude of 80% of the total fitted amplitude is observed for the fast

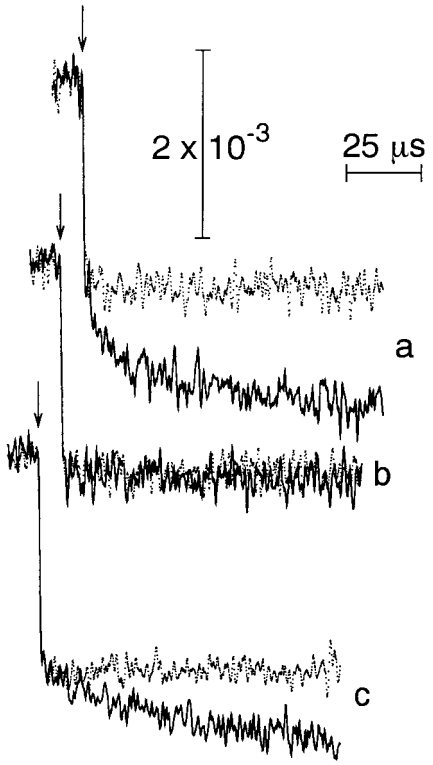

FIGURE 4: Flash-induced absorption changes at 580 nm observed for photosystem I complexes from *S. elongatus* in the presence of flavodoxin semiquinone from different cyanobacteria: (a) PSI from wild-type *S. elongatus* and flavodoxin from *Synechocystis* sp. PCC 6803, (b) PsaE-less PSI from the psaE-less mutant ES/8 of *S. elongatus* and *Synechocystis* sp. PCC 6803 flavodoxin, and (c) PSI from wild-type *S. elongatus* and flavodoxin from *Synechococcus* sp. PCC 7002. Experiments were performed in a manner similar to that for the experiments depicted in Figure 3 but with a higher number of scans (60 for trace b and 40 scans for traces a and c). Dotted lines represent control experiments with the same PSI in the absence of flavodoxin. All traces are scaled to an identical PSI concentration of 0.38 μM.

component, a value that exceedes that of wild-type PSI from *Synechocystis* sp. PCC 6803 (Table 1).

Heterologous Systems. For a more general characterization of the role of PsaE in flavodoxin binding, flavodoxin photoreduction was also investigated for PSI from the wild type and a psaE-deletion strain of *S. elongatus*. In Figure 4, results similar to those of Figure 3 are shown for PSI from *S. elongatus* using flavodoxin from either *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7002 as acceptors. For a comparison, the traces were scaled to a PSI concentration of 0.38 μM (see Materials and Methods). The photoreductions of the flavodoxin semiquinone from *Synechocystis* sp. PCC 6803 by wild-type PSI from *S. elongatus* (Figure 4, trace a) and *Synechocystis* sp. PCC 6803 are both biphasic processes and show qualitatively the same kinetic characteristics (see Table 1). In addition, like the results with the PsaE-less PSI from *Synechocystis* sp. PCC 6803, the fast phase of semiquinone reduction is completely absent for PSI from the *S. elongatus* psaE-less mutant (trace b) and the acceptor protein is reduced entirely by a second-order process (Table 1). These data indicate that PsaE is probably generally required for the binding of flavodoxin to PSI in cyanobacteria. However, when the same kinetics are analyzed on a longer time scale (experimental conditions as for measuring the first wave, but averaging of 60 individual experiments; see Materials and Methods), the rate of the slow phase is found to be slowed in the absence of PsaE to only 10% of the value observed for the wild type, an observation that is not detected for the homologous system (see Table 1). Furthermore, when the heterologous and homologous systems are quantitatively compared, it is immediately obvious that the relative amplitude of the fast phase observed for the wild-type photosystem from *S. elongatus* is already by a factor of 2 smaller than that for PSI from *Synechocystis* sp. PCC 6803 (Table 1). This effect is even more impressive for the photoreduction of flavodoxin semiquinone from *Synechococcus* sp. PCC 7002 by PSI from *S. elongatus* (Figure 4, trace c), where the fast phase is almost completely absent, except for a possible residual amplitude of less than 15% similar to the one discussed for the psaE-less mutant from *Synechocystis* sp. PCC 6803 (see above). Apparently, the semiquinone from *Synechococcus* sp. PCC 7002 has very little affinity for *S. elongatus* PSI, a finding that renders this system less suitable for studying the interaction between PSI and flavodoxin. In this system, flavodoxin reduction takes place mostly by the slow, second-order process with a rate of 1.9 ms^{-1} which is also somewhat smaller than but nevertheless comparable to the homologous system from *Synechocystis* sp. PCC 6803.

Chemical Cross-Linking. The PsaE-promoted interaction of flavodoxin with PSI was investigated by chemical cross-linking. Treatment of photosystem I complexes from *Synechocystis* sp. PCC 6803 or *S. elongatus* with the zero-length cross-linker EDC in the presence of flavodoxin results in the formation of five distinct cross-linking products between PSI subunits and flavodoxin (Figure 5, lanes 2 and 4). For both reaction centers, the resulting cross-linking pattern is very similar to the one obtained with PSI from *Synechococcus* sp. PCC 7002 that has been analyzed in detail (*22*). As described previously, these covalent adducts are mainly the result of cross-linking between the PSI subunits PsaC, PsaD, and PsaF with flavodoxin while the PsaE subunit is not cross-linked directly to flavodoxin under these conditions (*22*). However, almost none of these cross-linking adducts are formed when flavodoxin is cross-linked to PSI from the psaE-less mutants from both cyanobacteria (Figure 5, lanes 3 and 5). These results which confirm similar observations made with PSI from *Synechococcus* sp. PCC 7002 indicate that PsaE participates in the binding of flavodoxin to PSI, although this participation is not directly revealed by the observation of distinct chemical cross-linking adducts between the PsaE subunit and flavodoxin.

DISCUSSION

The kinetic characteristics of flavodoxin reduction by photosystem I from the *Synechocystis* sp. PCC 6803 wild type essentially confirm the data previously obtained for a similar process in *Synechococcus* sp. PCC 7002 (*22*). In both cyanobacteria, the oxidized form of flavodoxin is photoreduced by a simple bimolecular reaction in which a transient complex between the reaction partners is formed which is then followed by intramolecular electron transfer (k_{obs} = 600 and 500 s^{-1} for *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, respectively). In contrast, the reduction of the semiquinone is a biphasic process consisting of a fast first-order component ($t_{1/2}$ = 10 and 13 μs for *Synechococcus* sp. PCC 7002 and *Synechocystis* sp.

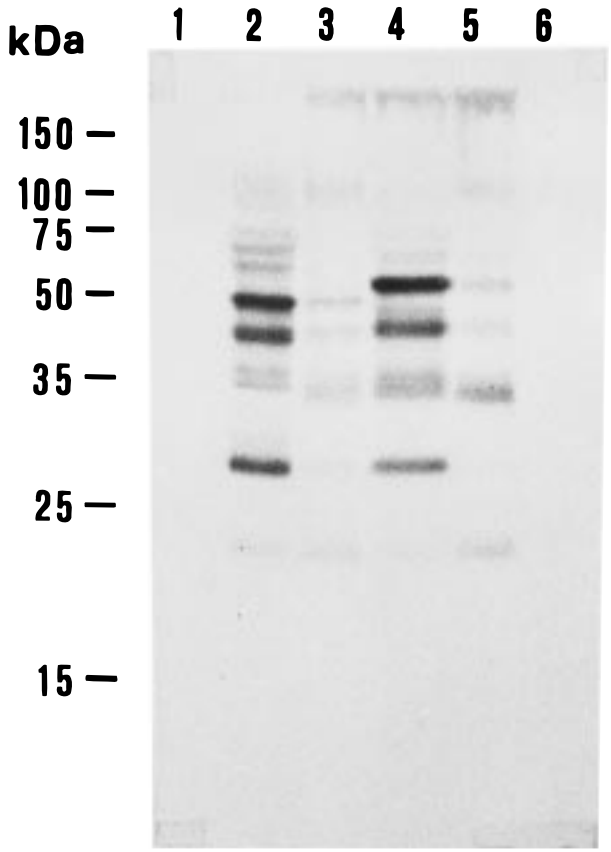

FIGURE 5: Immunoblot analysis of EDC-treated PSI preparations probed with antibodies against flavodoxin: lane 1, *Synechocystis* sp. PCC 6803 wild-type PSI; lane 2, *Synechocystis* sp. PCC 6803 wild-type PSI and flavodoxin; lane 3, *Synechocystis* sp. PCC 6803 PsaE-less PSI and flavodoxin; lane 4, *S. elongatus* wild-type PSI and flavodoxin; lane 5, *S. elongatus* PsaE-less PSI and flavodoxin; and lane 6, *S. elongatus* wild-type PSI. Bars to the left indicate the migration positions of recombinant protein standards (Sigma) whose molecular masses (in kilodaltons) are indicated (for details, see Materials and Methods.).

PCC 6803, respectively), which is interpreted as an electron transfer process within a preformed complex between flavodoxin semiquinone and wild-type PSI. The dissociation constants K_d of 2.6 and 2 μM were determined for *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, respectively. This first-order phase is then followed by a slower second-order process (second-order rate constants $k_2 = 1.7 \times 10^8$ and 2.2×10^8 M^{-1} cm^{-1} for *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, respectively) (for details, see ref *21*). The ability to discriminate between the two oxidation states of flavodoxin is thus likely a general phenomenon of PSI from cyanobacteria, and the deduced kinetic constants are likely very similar in most species. Nevertheless, great care should be taken when components isolated from different sources are used in studying this process as flavodoxin is apparently sensitive to structural changes in the photosystem and the affinity of the acceptor protein in its semiquinone form may vary considerably between photosystems from different organisms. In extreme cases, such as the reduction of flavodoxin from *Synechococcus* sp. PCC 7002 with PSI from *S. elongatus*, the affinity may be so low that the tight complex formation between PSI and the semiquinone that is observed when both reaction partners originate from the same organism may in fact be no longer detectable.

Fast first-order phases indicative of electron transfer reactions within preformed, tight complexes between PSI and flavodoxin semiquinone are also drastically diminished, or even fully absent, for semiquinone reduction in the presence of PSI from the psaE-less mutants of both *Synechocystis* sp. PCC 6803 or *S. elongatus*. The corresponding amplitudes which make up about 60% of the total amplitude of the process in the case of wild-type PSI under standard conditions are so drastically reduced that this phase can no longer be unambiguously resolved by kinetic fits of the absorption changes elicited from the PsaE-less PSI. In addition, the amplitude of the fast phase can be almost fully recovered by in vitro reconstitution of isolated PsaE to the mutant photosystem, a clear indication that the PsaE subunit is required for high-affinity binding of flavodoxin semiquinone to PSI. However, the analysis of the kinetic data for semiquinone reduction by the PsaE-less PSI precludes the conclusion that the binding affinity of PSI for flavodoxin semiquinone is completely zero. When the traces are fitted with only one kinetic component, a small, slightly concentration-dependent residual amplitude remains which may represent in part the residual amplitude of a fast phase. This remnant amplitude, however, is too small to allow either its conclusive interpretation or the reliable determination of a dissociation constant.

The comparison of the interaction between flavodoxin and PSI from the wild type and the psaE-less mutants using the chemical cross-linking approach shows that the absence of the PsaE subunit results in the almost complete disappearance of all major cross-linking species that are usually observed between flavodoxin and the cytoplasmatically exposed PSI subunits of the intact reaction center. This observation may indicate that flavodoxin is likely more loosely attached to PSI when PsaE is missing. In the presence of PsaE, flavodoxin is probably fixed in one distinct orientation on PSI to approach the terminal iron−sulfur centers F_A/F_B as closely as possible. Thereby, the protein establishes several close and distinct contacts with charged domains on the other extrinsic PSI subunits which must be at least temporarily close enough to allow a cross-link. In the absence of PsaE, however, flavodoxin may likely be allowed to approach F_A/F_B with a greater degree of freedom; thus, several orientations of flavodoxin on PSI are possible, and the contact to the other peripheral PSI subunits is no longer tight enough for an efficient cross-link.

Except for the fast phase which is lacking in the reduction of the semiquinone, PSI from the psaE deletion mutant and from the *Synechocystis* sp. PCC 6803 wild type behave very similarly. Both photosystems reduce both forms of flavodoxin with comparable efficiencies, and the calculated kinetic constants for the reduction of the oxidized flavodoxin and the slow phase of the second wave are in the same range for both types of reaction centers. The obtained constants vary by less than a factor of 2 in magnitude, variations that can also be readily observed for different preparations of PSI from the wild-type cyanobacteria (see Table 1). These results show that, although PsaE is involved in the binding of flavodoxin in its semiquinone form, the absence of this subunit has almost no effect on the overall amount of flavodoxin reduced by PSI. PsaE is therefore not essential for an efficient forward electron transfer from PSI to flavodoxin. This conclusion is in full accordance with the

fact that psaE deletion strains from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 show normal growth rates under conditions of iron limitation, conditions under which flavodoxin becomes the main electron acceptor for PSI (*22*−*31*). Similarly, the analysis of the photoreduction of ferredoxin by PSI from a psaE deletion strain of *Synechocystis* sp. PCC 6803 (the same mutant was used in this study) revealed that the affinity of PSI for ferredoxin is severely reduced when PsaE is missing, a defect that could be reversed upon reconstitution (*28*). The efficiency of ferredoxin reduction, however, was not significantly affected by the absence of PsaE (*28*), and the growth rates of psaE deletion strains from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 show no significant deviations from those of the wild-type strains under photoautotrophic conditions (*28*−*31*). Taken together, these studies therefore indicate that PsaE plays a comparable role in the photoreduction of both ferredoxin and flavodoxin by photosystem I. The PsaE subunit is involved in the high-affinity binding of ferredoxin and flavodoxin by PSI, and the reaction mechanisms are changed when this PSI subunit is missing. Nevertheless, the subunit is not required for an efficient forward electron transfer from photosystem I to both acceptor proteins.

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